Reply Exhibit K

Dear Dr. Yates,

After carefully considering and responding to the reviewers' comments, we are resubmitting the manuscript entitled "Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay (AEM01306-08 Version 1) to Applied and Environmental Microbiology. We have retitled the MS in order to respond to one reviewer's concern about terminology to: **Identification of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay**

We thank you and the reviewers for careful consideration of the manuscript and constructive criticism. The improvements to this version include the addition of more nontarget fecal samples and reference water samples to further support the reported specificity of the assay. **Like many MST applications, this one addresses a particular question in a particular geographic area.** We have taken pains to point this out more clearly in the manuscript. There is a strong probability that this method will be applicable to other geographic areas, but we have not tested that hypothesis in this work. The intrinsic value of this study is the application of MST principles to answering a crucial question in a given watershed: can we identify a poultry-specific DNA marker, develop a robust QPCR assay for it, and utilize it to quantify fecal contamination from poultry in the watershed?

A detailed accounting of the response to the reviewers' comments is given below. Because the manuscript has been extensively revised, we have summarized some of the responses to the minor comments, and have submitted a merged, compared document in which changes to the manuscript are tracked. Once again, we thank you for the time you are taking to handle this manuscript.

Dear Dr. Harwood:

Comments on your revised manuscript have been received from three members of the editorial board or ad hoc reviewers. The reviewers expressed a number of concerns about the manuscript. These include questions regarding the specificity of the markers for chickens (as described by Reviewer 1), the lack of some controls (as described by Reviewer 3), and the lack of sufficient data to "validate" the markers for other applications (See comments of Reviewers 1 and 2). In addition, it was felt that the presentation of the material was inadequate, and in some cases inappropriate, for a scientific journal. For these reasons, and the reasons in the attached reviews, I am unable to accept your manuscript for publication. The reviewer comments are attached and I believe that they will be helpful to you. Your interest to publish in Applied and Environmental Microbiology is very much appreciated.

REVIEWER 1:

Comment: There are three issues with respect to the specificity and general applicability of the markers that are of concern. 1. Litter is commonly the waste produced by broiler farms with birds running around on bedding, but liquid waste is commonly generated by egg producing farms with battery-fed hens. How could the marker developed here detect litter but not liquid chicken waste? This might not be an issue in this particular watershed, but certainly could be elsewhere depending on what kind of agriculture is being undertaken.

RESPONSE: The liquid waste from egg-producing birds was not considered in this study because, in contrast to the soiled litter produced by broilers, it is not land-applied on fields and has therefore has much less potential for impacting water quality. Furthermore, the great majority of birds produced in the Illinois River watershed (IRW) are broilers (~92%) while only about 3% are layers. These factors make the litter the major potential impact on water quality in the IRW. The specific geographic area covered by this study is emphasized in the abstract (L 31-32)

Comment 2. The amplification of a goose and a duck sample with the 'litter-specific' primers suggests that avian species in general may be detected.

RESPONSE: The goose and duck sample contained very low levels of the biomarker, in contrast to litter containing up to millions of copies per g. We know that these levels were very low because (1) nested PCR was required to detect it, (2) QPCR could not detect it in these samples and (3) nested PCR could only detect the biomarker in one of two replicate duck and goose samples. If the extremely sensitive nested PCR method could barely detect the marker in feces (one replicate only of one composite sample), there is a vanishingly small chance that it could be detected once diluted in water. Most of the MST markers in use today, e.g. the *esp* gene of *Ent. faecium* and the human *Bacteroidales* marker, experience some low level of cross-reactivity with the feces of non-target animals, yet they are still useful for determining sources of fecal pollution. Two new paragraphs have been added to the discussion that tackle the issue of specificity in molecular analyses used in environmental work (324-345)

Comment 3. The widespread applicability of the markers needs to be tested with samples from outside the constrained study area. Based on these considerations, how can the authors conclude with confidence that a given water sample was not impacted by broiler chickens, layer chickens, migratory birds, or resident birds? Are the authors confident that these markers would be useful for investigators working in watersheds that have these potential multiple fecal sources?

This study was designed to test the applicability of the assay in a geographically constrained area. Because of the severe environmental impact that land application of poultry litter COULD be causing, it was feasible to design a marker for poultry litter particularly for this area. The marker may well be applicable elsewhere, but the establishment of geographic range was not the goal of this study.

The levels of the LA35 biomarker are extremely high in contaminated litter (10 million to a billion gene copies per g). Considering the dilution factor that occurs when fecal material is washed into receiving waters, it would require a large amount of material with high concentrations of the target to be detected routinely. Reference water samples collected in areas removed from poultry farming or land application have also been added to provide evidence that unimpacted waters do not contain LA35.

Furthermore, environmental water quality studies should not make conclusions from isolated samples, rather, many samples should be collected and a weight of evidence approach must be used for data interpretation. Some error in any field test is inevitable; it is the responsibility of the investigator to interpret any test in the light of all the evidence. A discussion of this concept has been added (L 340-345)

Comment :There are numerous minor spelling, punctuation, inconsistencies in units of measure, and formatting errors in the text that need to be corrected. The location of commercial suppliers

needs to be provided. The citation list needs to be carefully reviewed for errors and edited to fit AEM format.

RESPONSE: We apologize for these errors and have worked carefully to correct them.

Comment: Running title: poultry

RESPONSE: QPCR assay for poultry fecal source tracking

Comment: l. 72. Not sure if it is necessary or advisable to mention the legality of the situation.

RESPONSE: Reference to the litigation has been removed from this section and appears only in the acknowledgments.

Comment: l. 77 and thereafter. Change British units of volume and length to SI.

RESPONSE: Corrected

Comment: l. 88. Beef pats, rather than scats.

RESPONSE: Scats has been revised to pats when referring to beef fecal samples throughout the manuscript

Comment: l. 115. ..on ice..

RESPONSE: Suggested text has been added.

Comment: l. 142 .. E. coli genus-specific.

RESPONSE: Removed and corrected to "E. coli-specific"

Comment: l. 186. plate read?

RESPONSE: Text has been revised to state a "fluorescence read" rather than plate read.

Comment: l. 210. the authors will have to present the results of the T-RFLP in figure or table. How many bands, what molecular weight, etc.

RESPONSE: To avoid unduly lengthening the manuscript, T-RFLP figures have been provided in supplementary material (Supplementary Figure 1)

Comment: l. 216. You mean the size of the plasmid insert in each clone was determined to see if it corresponded to the restriction fragment of interest?

RESPONSE: This paragraph has been revised for clarity and now reads:

Clone libraries. Clone libraries were constructed from the genomic DNA originally extracted from soil and litter samples in order to isolate clones containing the DNA sequence corresponding to candidate T-RFLP peaks. Amplified 16S rRNA fragments obtained using primers targeting the domain *Bacteria* [8F-907R (18, 26)] or *E. coli* [V1SF-V3AR (46)] were used to construct clone libraries. The *Bacteroidales*-specific primers were not used for library construction because no candidate targets were identified from T-RFLPs of this group. The template DNA for PCR was purified genomic DNA pooled from extractions of five subsamples for each clone library. The manufacturer's instructions for the TOPO ® Cloning Reaction kit (Invitrogen) were followed. Clone libraries were screened for the candidate target sequences by subjecting three hundred *E. coli* TOP10 colonies containing recombinant plasmids to T-RFLP as above, and identifying clones that contained one of the candidate peaks. The insert in each plasmid was sequenced….

Comment: l. 251. a line of evidence for fecal contamination, not of human health risk.

RESPONSE: Removed

REVIEWER 2:

Comments:

The subject manuscript describes a research to identify and validate a poultry-specific marker for use in fecal source tracking. Putative markers were identified through t-RFLP, sequenced, and primers developed for fragment detection by PCR. Sensitivity and specificity of the PCR markers were measured against fecal pools in the local setting (Oklahoma). Field validation was attempted by applying the candidate PCR assay to water samples in which poultry contamination was anticipated.

This reviewer takes no issue with the approach used to develop the host-associated marker nor the validation of the assay through sensitivity and specificity testing. The procedures followed in this regard represent the state of this rapidly evolving science. On the other hand, the information presented as field validation was, in fact, a field application and provided little information to support the utility of the marker assay for field application. The efforts described represent an uncontrolled trial from which no conclusions regarding the utility of the assay can be extracted. This critique does not necessarily mean that the authors' conclusions regarding sources of fecal contamination in the study area are unfounded, rather that the efforts did not support the main thrust of this manuscript as regards to assay development and validation. See main comments for more specifics.

This submission is stylistically weak. The authors use biased language, phrases frequently are redundant, important information sometimes is lacking, and presentation of information sometimes lacks sufficient organization. In sum, this reviewer feels that the authors provided an insufficiently finished product to the journal for consideration. See major comments for examples of stylistic flaws in the manuscript.

Major comments:

1. The report is not sufficiently finished. These are not infrequent, minor style issues but are pervasive and make the manuscript exceedingly difficult to understand (detailed in minor comments). The references are not stylistically consistent.

RESPONSE: We apologize, and have taken pains to correct these deficiencies.

2. Frequently missing information: What cultivation media were used to grow the indicator bacteria (line 117);

RESPONSE: Methodology added lines 128-131:

"Fecal coliforms and *E. coli* were enumerated by multiple tube fermentation (MTF) and calculation of the most probable number according to SM-9221F or SM-9230 (3). MTF tubes containing *E. coli* were identified using broth cultures supplemented with (MUG) (SM-9221F)".

2. how long were samples in transit before they were received and processed within 12 hours (line 131);

2. where is the accession information for the clones (line 141 or 197);

RESPONSE: The accession # for pLA35 is FJ462358, and appears at the end of the Methods section. Accession numbers for the other sequences are FJ469977 7 (Pantoea ananatis clone SA15), FJ469978 (Kineococcus sp. clone SA19) and FJ469979 (Uncultured bacterium clone SB37). These numbers are now in the text at the end of the Methods section.

2. how were the products sequenced (before line 149);

RESPONSE: This information has been added in the Methods under **Clone libraries:** "Two primer sets were used for double coverage of the sequence; one with sites located on the plasmid (M13F and M13R) (32) and one with sites located within the insert (388F and 519R) (1, 27)."

2. what constitutes weak amplification in a nested qPCR (line 229; assumed qPCR because of footer in table 4);

RESPONSE: This ambiguous term has been removed, and in the paragraph under the new heading **Evaluation of candidate biomarker specificity** we have written:

"Two of 36 composite fecal samples gave false-positive results in the nested PCR assay, i.e. one duck and one goose sample collected outside the watershed. Only one of two duplicates of each of these samples amplified by nested PCR, indicating very low levels of the target sequence. The presence of the LA35 sequence was confirmed by melt curve analysis of qPCR products and DNA sequencing; however, the qPCR assay was unable to detect the cross-reactive goose and duck sequences (data not shown). "

2. six putative markers were identified with assays developed (asterisk in Table 1), yet data for only 4 are presented (tables 2 and 3);

RESPONSE: Thank you for catching that error – primers were developed for only four T-RFs, as two of the E. coli T-RFs had no unique regions for primer development. Table 1 has been modified and the results have been clarified:

All three of the candidate clones from the *E. coli* library were isolated (T-RF 496.0, 498.9 and 500.8), but only three of the six candidate clones from the *Bacteria* library were isolated (T-RF 142.9, 147.3 and 158.9). Inserts containing the T-RFs of interest (Table 1) were sequenced and PCR primers were developed for those regions of the 16S rRNA gene that contained unique sequences (Table 2). Two of the three *E. coli* T-RFs identified as candidate biomarkers (Table 1) did not contain mismatches between the sequence of interest and the sequences of closely related organisms identified in a BLAST search and therefore were not considered further.

2. what were the optimization steps used in development of the qPCR assay (line 235)

RESPONSE: This phrase has been amended, as all steps for method development are included in the methods:

Quantification of LA35. A SYBR green qPCR protocol was developed using the LA35F/LA35R primer set.

3. Quantitative reports of marker concentrations are compromised by the lack of a recovery efficiency control in these samples. Recovery efficiency can vary wildly.

Many qPCR articles published very recently in top journals do not include recovery efficiency controls (e.g. Ahmed et al 2008, Env Microbiol Aug 14 epub ahead of print; Shanks et al 2008 Appl Env Microbiol 74: 745). While these controls may be desirable for many applications, the lack of these controls does not compromise our conclusions here.

4. The conclusion of the abstract, "potential for determining fecal source allocations for TMDL programs" is not supported by the manuscript. TMDL programs almost universally rely upon E. coli or fecal coliforms to indicate the total amount of fecal contamination. It follows that a fecal source allocation for TMDLs will not be possible without a strong relationship between E. coli density and marker concentration in poultry litter. No such relationship was demonstrated in this research (Figure 3). The authors correctly report this fact in lines 289 and 290, contradicting the statement in the abstract. More samples will not alleviate this condition unless marker concentration and fecal indicator density in reference material are shown to be related with a slope of 1:1 as concentration increases (counter to what is shown in Figure 3, slope 0.6:2.5 for enterococci and weak relationship for E. coli).

RESPONSE: The abstract conclusion has been rewritten and the reference to TMDL programs has been removed:

"The biomarker will contribute to quantifying the impact of fecal contamination by land-applied poultry litter in this watershed"

5. The analysis of relationships between E. coli or enterococci density and putative poultry marker concentration in water is incomplete. In any given water sample, fecal contamination from any number of sources may be present. Thus, any validation for a relationship between poultry marker and fecal indicator must take into account the expected level of poultry contamination. Importantly, the ratio of marker to indicator would be relatively low for water with lesser poultry-origin contamination (bulk river water, especially upstream from poultry-amended fields), and relatively high with concentrated poultry contamination (as expected in runoff from litter-amended fields). Lumping all water samples, without regard to the expected level of poultry-origin contamination, and looking for a direct correlation is not particularly informative and does not constitute validation. Despite not presenting land-use information, the authors present (line 285) the suggestion that land use and level of contamination by poultry litter are correlated.

RESPONSE: In order to focus this manuscript on biomarker development we have removed data pertaining to surface waters with the exception of edge-of-field runoff directly from field that had received poultry litter spreading. A more comprehensive manuscript that includes supporting data from additional testing methods on surface and ground water samples will be submitted at a later date (Table 4).

6. Correlation of poultry marker with fecal indicators (line 252) does not provide any evidence of human health risk. The relationship of fecal indicators with human health risk was developed at sites contaminated primarily with human waste (Dufour's publications, 1984 and 1986). This relationship is not expected to be the same for water contaminated with feces from nonhuman sources.

RESPONSE: This sentence has been removed.

Minor comments: (**Authors' Note**: the minor comments that deal with non-technical points have been dealt with; however we will not detail their outcome in the response because the manuscript has been extensively revised. In these cases "OK" denotes that we have attended to the comment.

39 – biased language

RESPONSE: Although there was no intent to provide biased commentary, in deference to the reviewer's opinion we have modified the sentence to read: "Land application is the prevalent disposal method for poultry litter in the U.S., although alternative disposal practices are available (8, 22)."

40 – pollution contaminating is redundant OK

44 – space missing between text and reference OK

46-49 – this description of the state of regulatory fecal-indicator bacteria in the United States likely will confuse both native and international readers. While recognizing that the focus area is Oklahoma in the United States, generalize the content to be relevant to an international audience. Suggest three sentences that summarize the state of regulations at the local, national, and international scale. You already have state and national to work from, for international consider WHO documentation for E. coli density criteria in bathing waters.

RESPONSE: The passage has been rewritten for clarity as follows:

"In Oklahoma, the site of this study, the recognized indicator organisms are fecal coliforms, *Escherichia coli* and enterococci (Oklahoma Administrative Code, Title 785, Chapter 46). The U.S. EPA recognizes *E. coli* and enterococci as indicators of recreational water quality in fresh waters (47). The World Health Organization recognizes enterococci as the indicator organism (or index organism) for recreational water quality (WHO 2003).

50-53 – run on sentence, awkward and difficult to understand OK

53-56 – run on sentence OK

58 – MST markers typically are not proposed as alternatives to monitoring fecal-indicator bacteria. One exception is general Bacteroidales in the USEPA (Wade) epidemiological study. MST markers more commonly are proposed to augment information about total contamination levels (fecal indicator bacteria densities) with information about sources (presence or relative abundance of fecal contamination from a targeted source). OK

64 – The compound noun "Host marker specific targets" is complex to the point of being nearly nonsensical. OK

67 – Misspelling, "Bacteroides" OK

69 – Two non-Bacteroides markers with purported specificity to poultry (CB-R2-42, CP1-25) were proposed in the cited article. OK

72-73 – Relevance of this statement to the content of the article. This information is presented already, appropriately, in the acknowledgements.

RESPONSE: Removed from introduction and shown only in acknowledgments

75 – This section really needs to be split into paragraphs. Recommend paragraph breaks at line 80, 84, 101 to enhance readability. OK

80 – How many fields were sampled? Where were they?

RESPONSE: The sentence has been clarified (3rd para of Methods)

" Ten litter application areas in separate fields (soils) within the watershed were sampled by collecting 20 subsamples on a predetermined grid across a uniform area of one to ten acres."

81 – Predetermined grid pattern is redundant. OK

82 – The 0 to 2-inch layer

RESPONSE: Changed to read: "The zero to two inch fractions from six inch soil cores…"

83 – Did vegetation, feathers and rocks make it through the 2 mm sieve?

RESPONSE: No, modified for clarity

87 – Two independent duplicate samples is redundant. OK

88-89 – The summation of 200 cattle pats is not needed after the prior description.

RESPONSE: Because the description of fecal sample collection and compositing is necessarily detailed, we would like to retain the summation to remind the readers that many animals were tested through this procedure.

90 – Independent duplicates is redundant 95, 96 – 2 duplicate samples is redundant OK

97 – Comma needed after each) OK

102 – A churn splitter is not a sample collection method

RESPONSE: No longer relevant, as all data regarding river, lake and groundwater samples has been removed from the text.

108 – Filtration is only part of the processing samples underwent. Suggest "filtered to recover total DNA and aliquoted for most-probable-numbers analysis of fecal-indicator bacteria." Were there other processes as well?

RESPONSE: This section has been revised for clarity (L 117-129)

114 – All samples; it seems likely that feces, soil, and litter samples also were shipped to the laboratory for filtration.

RESPONSE: Fecal, soil and litter samples were processed directly, as explained in L 114-116 and L 135-138

117-121 – This method is very imprecise.

RESPONSE: We believe that the author is referring here to multiple tube fermentation/most probable number enumeration methods for indicator bacteria. Standard methods were used for this culture-based analysis. It could well be argued that culture-based methods for assessing bacterial populations are inherently imprecise. Nevertheless, these methods are approved for water quality analysis and are used by regulatory agencies across the country.

125 – Suggest inserting "further", as "was further purified" for clarity. OK

130 – Terminology. Likely the authors meant re-dissolved (if traditional ethanol precipitation) or, in line 129, captured and washed (if spin filters were used). OK

132-3 – Were Supor filters frozen to facilitate shattering? Yes - OK

142 – Perhaps from lack of experience with the cited primers, this reviewer is at a loss to understand how the E. coli-specific primers are described as genus-specific as well as how a non Escherichia clone (T-RF 500.8) was derived from the PCR products.

RESPONSE: T-RF 500.8 was identified as a *Pantoea ananatis* sequence, which is closely related to *E. coli* and is also a member of the *Enterobacteriaceae*. Finding a sequence from this organism after amplification with these primers is therefore not surprising. This has been clarified in the text in L 279-285:

"The one *E. coli*-like sequence for which unique primers could be designed (Table 2) represented a *Pantoea ananatis* strain, which, like *E. coli,* is a member of the *Enterobacteriaceae* (Brady et al 2008). Three primer sets derived from the universal bacterial library were developed for a *Brevibacterium sp.*, a *Rhodoplanes sp.* and a *Kineococcus sp.* (Table 2).

144 – Extraction should be the noun, extract. OK

187 – Units. The qPCR standards are (oddly) in units of ng/uL in the materials and methods but in units of copies/uL in Figure 2.

RESPONSE: The text has been revised to coincide with Figure 2, but also defines how ng/ul were converted to copies/ul.

190 – Since these are plasmid-based standards (line 187), and not based on cultivated Brevibacterium, then the assumption should be one insert copy per plasmid?. There appears to be little reason to extrapolate from copy number to genome number in the original sample. Question the use of the term "gene" for this DNA sequence.

RESPONSE: This section has been completely rewritten as follows:

"DNA standards ranging from $3.1*10^{-8}$ to $3.1*10^{-1}$ ng·uL^{-1} were prepared from serial dilutions of purified pLA35 and used to develop the standard curve. Plasmid copy numbers were calculated by measuring DNA concentration spectrophotometrically and converting from ng DNA·uL^{-1} to plasmid copy number (calculated weight of $4.9*10^{-18}$ g per plasmid). Assuming one gene copy per plasmid, the DNA standards ranged from ~3 to $3.1*10^{7}$ gene copies per PCR reaction."

195 – suggest substituting "and" for "were." OK

210 – The statement about "the two litter and two soil samples" makes it appear that those were the only samples in the study. This is counter to the presentation of sample collection from 10 poultry houses in line 75.

RESPONSE: This has been rewritten in the methods section as shown below.

"**T-RFLP analysis.** In order to identify candidate targets for development of a poultry-specific biomarker, extracted genomic DNA from two fecal-contaminated poultry litter samples and two soils samples that had received land application of poultry litter was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled primers for T-RFLP."

214-216 – Information belongs in Methods OK

227 – Suggest offsetting LA35 with commas for clarity. OK

229 – Were the assays developed accomplished by end-point PCR or by quantitative PCR? Table 4 footnote a says qPCR. If so, what constitutes a weak amplification?

RESPONSE: This has been rewritten on pg 14 under the heading **Evaluation of candidate biomarker specificity.**

"Only LA35, the *Brevibacterium* sp. clone, displayed the specificity to be further tested as a biomarker for poultry litter (Table 3). In the nested PCR assay, primers LA35F/LA35R did not produce products from any fecal samples with the exception of one duck and one goose sample collected outside the watershed. Furthermore, only one of two duplicates of each of these samples amplified, indicating very low levels of the target sequence."

235 – Optimization steps not listed in Methods.

RESPONSE: All steps for method development are now included in the methods:

251-254 – Run on sentence leads to ambiguity about whether "these organisms" (line 234) refers to regulatory fecal indicators or Brevibacterium.

RESPONSE: This sentence has been removed.

254 – The citation to a MST review article is non-ideal to support the concept of fecal indicator bacteria regrowth in the environment.

RESPONSE: This sentence has been removed.

272, 272 – Clarify whether the intent of source tracking is to address eutrophication, recreational use impairment, or both.

RESPONSE: We are not sure what this comment refers to. The sentence that starts on line 272 of the original MS is "As poultry litter is land-applied as a disposal practice (19, 33, 35), it was important to identify a marker that could survive the process of deposition on bedding and spreading on fields."

274 – Biased language. There is no sanitary criterion for soil that would justify the use of "contaminated."

RESPONSE: The term "contaminated" was meant to discriminate between clean, unsoiled litter and used litter that is soiled with feces. The term "contaminated" has been removed and the terms "used" or "fecal-soiled" have been applied throughout.

278 – Format of reference citation (26) OK

279 – Suggest further differentiating this study from the cited study by specifying that the cited study used fresh chicken feces as starting material, whereas this study used "aged" material that is more likely to actually contaminate a water body.

RESPONSE: The suggested differentiation has been made on pg 18 as shown below:

"Our rationale was that the persistent DNA and/or organisms found in the litter and soil were more likely to reach a water body and act as an effective tracer than targets that do not persist outside the host. This strategy for marker identification is in contrast to the work by Lu and colleagues (30), in which chicken feces were used as the starting material for a genome fragment enrichment method for marker identification."

279 to 283 – The comparison is not valid and should be removed or expanded upon. This study used fecal composites, whereas the cited study used samples from individual chickens. There is no evidence that the marker in this study is more broadly distributed in individual poultry than are the markers in the cited study.

RESPONSE: The reviewer is referring to this statement: "Based on the PCR assays developed from clone libraries of the genome fragments, 6 to 40% of the chicken fecal samples collected from a wide geographic region contained DNA that could be amplified by the various assays (26). In comparison the LA35 biomarker was found in all the poultry litter samples tested, although it should be noted that all of the samples were collected in the Oklahoma/Arkansas region."

There was no intent here to extrapolate our results to individual birds, and we find it difficult to understand why the reviewer interpreted it this way. However, we have made the following clarification in the discussion: (italics appear in this letter only, not in the manuscript)

"Based on the PCR assays developed from clone libraries of the genome fragments in that study, 6 to 40% of the *individual* chicken fecal samples collected from a wide geographic region contained DNA that could be amplified by the various assays (30). The LA35 biomarker was found in all the *composite* poultry litter samples tested here, although it should be noted that all of the samples were collected in the Oklahoma/Arkansas region."

301 – Ambiguous terminology, using separate to mean independent but the samples actually were composite samples (not from separate, as in from individual animals).

RESPONSE: This section has been revised and no longer contains this sentence.

301 – Space missing between against and 10 See above.

302 to 304 – No land-use data presented to support the expectation of variable concentrations of biomarker. This issue needs to be addressed throughout the paper.

RESPONSE: In order to focus the manuscript we have presented environmental data from only land-applied fields and edge of field runoff samples. Thus there is no longer expectation of variable concentrations in this work.

306 – Suggest sentence break " …concentration. The correlation was …" for clarity

No longer applicable

307 – Suggest "… Brevibacterium spp. Based marker for microbial …" OK

308 – "… among the first … methods …" OK

308 – Though the marker is quantifiable, the evidence presented in this paper does not indicate that use of the marker is sufficient to quantifiably track poultry fecal sources in environmental waters, at least in the sense of 10% of E. coli or nutrients in this water body came from poultry-derived fecal contamination.

RESPONSE: In order to focus the manuscript, the scope has been narrowed and the summary has been revised as shown below. The broader environmental sampling program will be published in the context of a weight of evidence approach in a future manuscript.

'In summary, a biomarker for used (fecal-soiled) poultry litter was identified and a quantitative PCR assay was developed for the LA35 biomarker. The assay displayed high sensitivity and specificity, and had a method detection limit that is low enough to be applicable for environmental water and soil samples. The significant, positive correlations between LA35 and fecal indicator bacteria concentrations suggest its connection with indicator organisms used to regulate recreational water quality. The research presented herein is the first identification of a

Brevibacterium spp. based marker for microbial source tracking studies and is among the first methods for tracking poultry fecal sources in environmental waters."

347 – Question whether the last three authors on this paper all have the same name. They do!

392 – Note Santo Domingo, not Domingo OK

Table 1. 20 profiles (5 subsamples*4 samples) were generated, yet only 19 appear in Table 1.

RESPONSE: This has been corrected in the methods, L 166-167

"Three to five subsamples of each poultry or litter sample were subjected to the procedure below."

It would be nice to order the t-RF and PCR reactions consistently in Tables 1-3. OK

Figure 2, how many replicates? Dynamic range? Matrix effects?

RESPONSE: This is now explained in the Methods, the Results and the Figure legend.

In Methods: "Triplicate measurements of each standard concentration were made."

"All samples were spiked with pLA35 and subjected to qPCR to ensure that the PCR was not inhibited. When necessary, template DNA was diluted to 10 $ng \cdot \mu L^{-1}$ to alleviate inhibition."

In Results: "A representative standard curve produced from amplification of the cloned sequence, with a detection limit of 6 $copies \cdot \mu L^{-1}$ (~30 copies per PCR reaction), is shown in Figure 2. The response remained linear up to the maximum concentration of $3*10^7$ copies per reaction."

Table 4 – the difference between a "detection" and a "quantification" is not sufficiently described.

RESPONSE: This has been clarified by comments following Table 4.

Figure 3 – The X-axis should be labeled biomarker, not Brevibacterium, to reflect the actual measurement. Ibid Figure 4 OK

REVIEWER 3:

Comments:

The aim of this study was to identify a poultry litter-specific biomarker, to determine its specificity against other fecal sources and define a 16S rRNA real-time PCR for quantifying the proposed biomarker in environmental samples. Authors defined properly the purpose of the study and used adequate procedures. However, control materials (samples) are missing in order to determine the feasibility of the approach to a real situation in the environment. The use of DNA standards for real-time PCR is necessary but they are just providing internal control for the molecular method but not for controlling the usefulness and the detection limits when applied on environmental samples.

Other comments:

Page 2 lines 30 -32. Please, consider to remove this sentence. It is not supported by the present study.

Page 4 lines 72 – 73. This sentence is not scientifically relevant. It is already indicated on acknowledgements. Please, remove it.

Page 5 line 78 - 82 (and over the rest of manuscript). As it is indicated on "AEM Instructions for authors", it is preferable to use the Système International d'Unités (SI). Please, follow instructions for authors.

Page 5 line 85. Please, consider to add ".... From groups of individuals (cattle, duck, swine and human sewage):" It could avoid citing Table 3 on text before than Table 1 and 2. Revise number of tables and their appearance order on the text.

Page 7 line 131. Volume of water samples is missing. It should be indicated to support detection limits of the performed analyses.

Page 7 line 142. Citation on text. Please, revise it all along the manuscript by following "AEM Instructions for authors".

Page 8 line 155. Bacteria should not be in italics. The term is not referring genus neither species. Please, revise it all along the manuscript (for instance, lines 171, 173, 213 and so on).

Page 9 line 167. Again, consider to revise numbering for tables.

Page 11 line 212. Again, consider to revise numbering for tables.

Page 11 lines 214 – 215. Please, move this sentence to M&M.

Page 11 lines 225. Was it nested PCR? If so, please, explain on M&M, it is not clearly indicated. For instance, qPCR (on M&M) is only reported to be used to amplify the 16S rRNA gene from Brevibacterium spp. DNA samples (lines 177 – 178). Moreover, what means Brevibacterium spp. DNA samples? Non-tergat environmental samples) or extracted DNA from a collection strains of Brevibacterium?

Page 12 line 238. Please, consider to add: ".... of extracted DNA when using clone plasmid DNA standards".

Page 12 lines 234 – 244. The detection limit of any new molecular methods for environmental use should be determined using DNA standards obtained similarly to DNA environmental samples (control materials). For instance, Brevibacterium spp. type strains culture suspensions prepared using a similar water matrix (and a serial of ten-fold dilutions for calibration) should be assayed following concentration and DNA extraction procedures used later for environmental samples, and next the defined qPCR assay. Such kind of internal control suspensions would provide the real detection limit of the new molecular when applied on environmental samples. Clone plasmid DNA standards are good control just for the qPCR analytical method. Have authors checked the proposed procedure with this kind of control samples?

Page 12 line 247. Figures 3 and 4 are not necessary to support the content of text. Both figures could be removed.

Page 12 lines 253 – 254. The evidence for regrowth of E. coli and other intestinal microorganisms is questioned by some researchers. Please, consider to remove the last sentence of the paragraph (….although there is ….into the environment (36)). It is not supported by the present work and is out of scope for the paper.

Page 13 line 256. "Validated" is not an appropriated term attending to the performed study. Please, consider the revision of this paragraph by checking ISO/TR 13843 (Water Quality – Guidance on validation of microbial methods) for the definition of validation and what it requests.

Page 14 line 298. Please, remove subheading by following "AEM Instructions for authors".

Page 22. Table 1. Please, avoid repetition of number of subsamples tested all along the columns. It could be solved by adding to the table heading "…had been applied. Number of subsamples containing T-RF of interst. n, number of subsamples tested". Then, remove this text on the table, put below Litter A (n=4), Litter B (n=5) and so on, and remove "n" values on each column by keeping without parenthesis the respective number of subsamples containing T-RF of interest. It would simplified the table.

Page 24. Table 3. Similarly to Table 1. Please, consider to chance Table heading to: "Specificity of the poultry litter biomarker nester PCR assay tested against fecal samples from within and outside the watershed. N, number of tested samples". Then, add a column (label, n) after the first column for the number of samples tested, and avoid the repetition of n on each column. Parenthesis and text on the table "Number of smaples tested" should be removed.

Page 26. Figure 2 is not necessary if it is commented on text.

Page 27. Table 4. The estimation of the corresponding number of cells/100 ml is suggested to be indicated. The reported concentration of biomarker could be moderate or low at point source in terms of cells. Bacteria which are alive or metabolically active could have easily 10E4 ribosomes (targets) by cell. To know the concentration of the proposed MST indicator at point source by using common units for most of microbial water indicators is convenient. It has been described that high concentration of MST indicators at point source are necessary otherwise they will be not feasible because they could not be measured after dilution and die-off on the environment.